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PRINCIPAL INVESTIGATOR: Sophie Thiolloy

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203-6869

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Sophie Thiolloy

E-Mail: sophie.thiolloy@vanderbilt.edu

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14. ABSTRACT
Bone metastasis is a common event during breast cancer (BC) progression. Matrix Metalloproteinases (MMPs) are often overexpressed in BC and play an important role in tumor progression. Metastatic BC is typically osteolytic and we hypothesize that specific stromal and tumor MMPs contribute to the growth and development of osteolytic lesions. To address the role of individual stromal MMPs in vivo, we used an intratibial model that recapitulates breast induced osteolysis. We demonstrated that stromal MMP-2 and MMP-7 are required for mammary tumor growth in the bone and the development of osteolytic lesions. However MMP-9 does not affect tumor growth and bone resorption in our model of mammary tumor-associated bone lesions. These findings emphasize the need to delineate the role of specific MMPs in breast induced-bone osteolysis to hopefully open the way for new therapeutics.

15. SUBJECT TERMS
Breast cancer, Matrix Metalloproteinases, osteolysis

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Introduction

In the United States, this year, 80% of the women who will succumb to breast cancer, will have evidences of bone metastasis[1]. The process of breast to bone metastasis illustrates the "seed and soil theory" which explains that certain tumors spread to specific organs depending on the complexity of the interactions between the tumor cells (seed) and their environment (soil)[2]. The skeleton is a common site for metastasis in many cancers such as breast, lung or prostate cancer. Breast to bone cancer metastases are typically osteolytic and induce bone destruction[3].

The MMPs are a family of enzymes that degrade the extracellular matrix and a variety of signaling molecules and cell surface receptors such as osteopontin, TNF-α, TGF-β and RANKL[4-6]. Thus, MMPs, by cleaving and/or solubilizing these functional factors, can modify the communication between tumor cells and the host microenvironment[7]. In the normal bone stroma, a number of MMPs have been detected in osteoclasts, the cells responsible for bone resorption, such as MMP-3, -9, -13 and –14[8]. Our understanding of the contribution of specific MMPs to pathological conditions such as breast cancer induced osteolysis is limited. The use of broad spectrum MMP inhibitors can decrease and even prevent breast tumor induced osteolysis in animal models[9-11]. However , the contribution of specific MMPs to the observed tumor growth and osteolysis remains to be determined. In the current project, we are investigating the role of specific MMPs in mammary tumor growth induced osteolysis and determine the molecular mechanisms through which specific MMPs contribute to this process.

Project

Accomplishment

Task 1. *Determination of the contribution of stromal MMP-2, -3, -7 and -9 to mammary tumor growth and osteolysis in the in vivo tumor:bone microenvironment.*

- **a.** Intra-tibial injection of luciferase tagged PLB1 (forms primary tumors, induces bone destruction and forms lung metastases) cells studies in MMP deficient (MMP-2, -3, -7 and -9) and wild-type FVB mice (months 1-24): familiarization with real time imaging modalities such as Xenogen's IVIS system for monitoring luciferase activity (months 1-6).
- **b.** Generation of *in situ* hybridization protocols for MMPs in bone tissue (months 7-30).
- **c.** Histological analysis of wild-type and MMP deficient mice intra-tibially injected with PLB1 cells (months 3-30) and immunohistochemistry and cytochemistry for the localization of tumor cells, bone cells and immune cells in sections from intra-tibially implanted tumors.
- **d.** Collation of the data and publications of the results (months 27- 36).

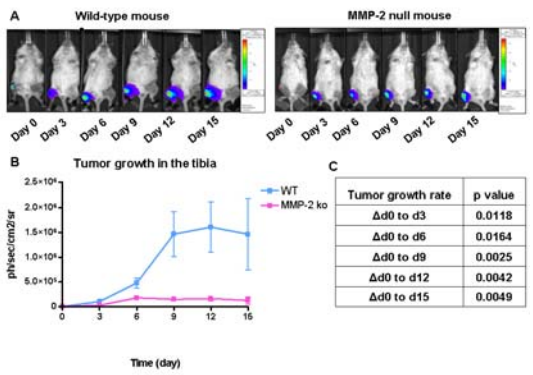

Figure 1. *Stromal MMP-2 increases mammary tumor growth in the bone.* We injected PLB1 luciferase cells into the tibia of FVB wild-type (n=11) or MMP-2 null mice (n=10) and image them over time (days 0, 3, 6, 9, 12, 15) using the IVIS system from Xenogen (A). The bioluminescent signal was quantified using the living image software (B). The tumor growth rate for each time point in the two groups of animals was calculated (C).

Our initial studies have focused on MMP-2 and MMP-9. To assess if stromal MMP-2 affects mammary tumor growth in the bone, we injected the murine breast cancer PLB1 cells into the tibias of wild-type (n=11) or MMP-2 deficient (n=10) FVB mice. This cell line has been isolated from the polyoma middle T antigen (PyMT) FVB mice. These mice develop spontaneous mammary tumors that recapitulate the pathology of human breast cancer[12]. Using this cell line allows for the use of fully immune competent FVB mice representing a more accurate human scenario. After injection of the PLB1 cells tagged with

luciferase, the growth of the tumor was quantified every 3 days by a retro-orbital injection of luciferin (105ng/kg). Photoemission from the tumor was quantified with the Xenogen IVIS™ imaging system (**Figure 1**). From day 3 onwards, we observed a significant difference in the tumor growth rate in the MMP-2 null mice compared to the wild-type animal ($p<0.02$, Student's *t* Test). The most striking difference in the tumor growth rate appeared between day 6 and 9 post-injection. To investigate further the effects of stromal MMP-2 on mammary tumor growth, we sacrificed mice (n=6 to 7) in each group at day 6 and day 9 after injection to perform immunohistochemistry analyses. Tumor proliferation and apoptosis in the bone was assessed respectively by phospho-histone H3 staining and terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphatase nick end labeling (TUNEL) staining. No difference was observed in both tumor proliferation and apoptosis in the two groups of animals (**Figure 2**).

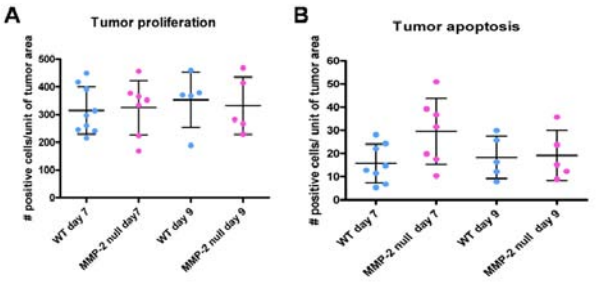

Figure 2. *Proliferation and apoptosis in wild-type and MMP-2 null mice.* PyMT-R221A luciferase cells were injected into the tibia of wild-type or MMP-2 null mice. At day 7 and day 9 after injection, mice were sacrificed and phospho histone H3 staining and apoptosis staining were performed on the injected legs. No significant difference in proliferation and apoptosis was observed between wild-type and MMP-2 null mice (A and B).

Immunohistochemistry does not allow us to accurately distinguish between tumor and host cells proliferation and apoptosis. Therefore, we decided to engineer a PLB1 cell line expressing both luciferase and DsRed2 allowing us to monitor tumor growth by luminescence imaging and tumor proliferation and apoptosis by fluorescence activated cell sorting (FACS). The luciferase-DsRed2 expressing PLB1 cells will be injected into green fluorescent protein (GFP) wild-type or MMP-2 null mice allowing us to distinguish by FACS analysis tumor versus host cells. To avoid any difference in the expression level of the two genes of interest (luciferase and DsRed2), we decided to use the LZRS-MS plasmid kindly provided by Dr Reynolds, Vanderbilt University. This expression vector contains an internal ribosomal entry site (IRES) allowing the same level of expression of the genes inserted upstream and downstream of the IRES. Proliferation will be assessed using an antibody specific to mouse histone H3 and apoptosis will be detected using a mouse specific antibody against cleaved caspase-3 (FACS). Host cells will be double GFP-histone H3 or caspase-3 positive, therefore distinguishable from tumor cells which will be double stained DsRed2-histone H3 or caspase-3 positive. We have engineered the wild-type and MMP-2 null FVB GFP expressing mice and we are currently working on establishing the luciferase-DsRed2 expressing PLB1 cell line.

To determine the role of host MMP-2 in mammary tumor induced osteolysis *in vivo*, we initially focused on osteoclasts and performed tartrate-resistance acid phosphatase (TRAP) staining (a marker of active osteoclasts). Using the Osteomeasure™ software from Osteometrics, the volume of trabecular bone and volume of tissue area were determined and we calculated a ratio of volume of trabecular bone to tissue and normalized this ratio for each tumor bearing tibia to the control tibia. A significant decrease in trabecular bone volume was observed in the wild-type mice compared to the MMP-2 deficient at day 9 post-injection (**Figure 3**). Using the same software, we also determined the ratio of number of mature osteoclasts per perimeter of trabecular bone and no significant difference was observed between the two groups of mice (**Figure 3**). To determine if the differences in tumor growth and bone resorption observed was due to a difference in the immune cell populations, we established by FACS the immunotype of both groups of mice in presence and absence of

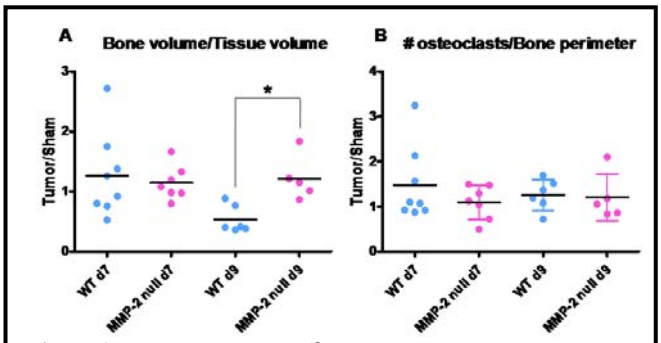

Figure 3. *Host MMP-2 contributes to mammary tumor induced bone resorption.* PyMT-R221A luciferase cells were injected intratibially into FVB wild-type or MMP-2 null mice. At day 7 and day 9 after injection, 6 to 7 mice in each group were sacrificed and histological analysis were performed (A). The ratio of trabecular bone volume to tissue volume was determined. At day 9 post-injection, a dramatic decrease in the ratio is observed in the wild-type animals compared to the MMP-2 deficient animals ($p=0.002$, Mann Whitney) (B). However, no difference was observed in the number of osteoclasts at the two time points.

tumor. We used specific immune markers such Gr-1 (neutrophils), B220 (B cells), CD4 (T cells) and F4/80 (activated macrophages) and no striking difference was observed in the different immune cell populations between the MMP-2 null mice and the controls. Task 1b will now be pursued to identify the cellular origin of MMP-2 in our tissue samples.

We also investigated the contribution of stromal MMP-9 in tumor growth and bone resorption in our *in vivo* model. We injected PLB1-luciferase cells into the tibia of FVB wild-type and MMP-9 null mice and tumor growth into the tibia was followed using bioluminescent imaging for 9 days. No difference was observed in the growth of the tumor between MMP- 9 deficient mice and control mice (**Figure 4A**). Using high resolution micro-computer tomography (CT) scanning technology trabecular bone volume was assessed in the tumor and sham injected limb at the end point of the study([**Figure 5A-B**). We are currently quantifying the number of osteoclasts present in tumor bearing legs of wild-type and MMP-9 deficient mice.

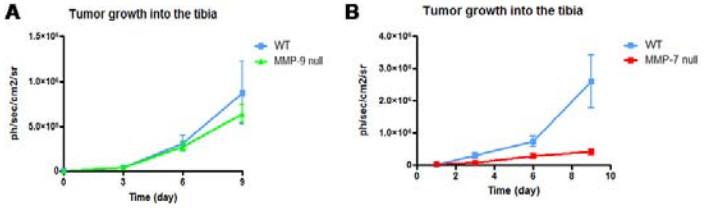

Figure 4: *Host MMP-7 but not MMP-9 contributes to mammary tumor progression in bone.* Real time imaging of intratibial growth of PyMT-R221A luciferase mammary tumor cells in Rag2$^{-/-}$ immunodeficient wild-type (n=5) or MMP-7$^{-/-}$ (n=10) female mice and in FVB wild-type (n=6) and MMP-9 null mice (=11). No difference in tumor growth rate was observed in MMP-9 deficient mice compared to the controls. However, the tumor growth rate was significantly slower in the MMP-7 deficient mice compared to the wild-type controls (Student's *t* test, p=0.025).

As MMP-7 deficient mice were not available in FVB background, we investigated the contribution of MMP-7 in tumor growth in the bone in the immunodeficient Rag2 null C57BL/6 mice, lacking mature B and T cells. Upon intratibial injection of the PLB1-luciferase cells, a significant slower tumor growth rate was observed in the MMP-7/-Rag2 null mice (**Figure 4B**). This decrease in tumor growth was concomitant with a reduced trabecular bone volume determined by CT scanning (**Figure 5C-D**). We are currently assessing the number of osteoclasts present in the tumor bearing limbs of wild-type and MMP-7 null mice using the Osteomeasure ^{TM} software.

We have now available in the laboratory MMP-3 deficient mice in the FVB background and studies will be performed as soon as sufficient mice will be available.

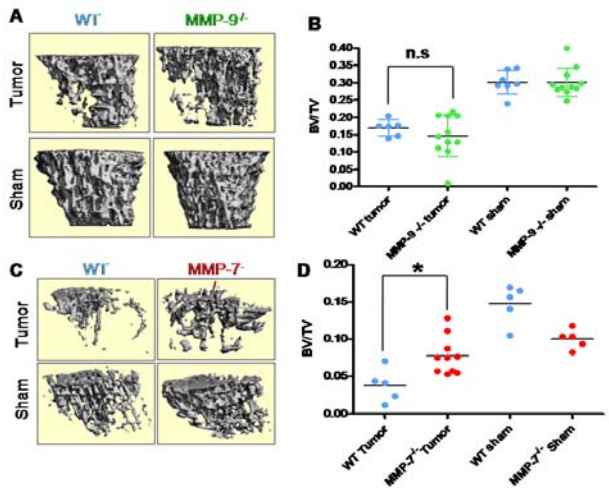

Figure 5. *Host MMP-7but not MMP-9 contributes to mammary tumor induced osteolysis.* Using high resolution micro CT scanning technology, trabecular bone volume was assessed in the tumor and sham injected (saline) tibias of wild type and MMP9$^{-/-}$ and MMP-7 $^{-/-}$ animals at the end point of the study (9 days post-injection) (A, C). Analysis of the BV/TV ratio revealed no difference between the tumor injected tibias of the wild type controls and MMP-9$^{-/-}$ deficient animals (B).However, MMP-7$^{-/-}$ deficient animals presented a decreased BV/TV at the end point of the study compared to the controls mice (One way Anova, p<0.05) (D).

<u>Task 2.</u> *Identification of the mechanism by which specific stromal MMPs contribute to breast tumor induced osteolysis using an ex vivo approach.*

- **a.** Generation and characterization of the *ex vivo* calvaria model (months 1-6).
- **b.** Isolation of MMP deficient calvaria and histological assessment of tumor induced bone destruction. MMP deficient animals to use will be identified in Task 1, part a (months 6-32).
- **c.** Collation of the data and publication of results (months 34-36)

The execution of this task relies on the use of an *ex vivo* calvaria model described by Ohshiba *et al*, 2003[13]. We have focused our initial effort on understanding how MMP-2 contributes to mammary tumor induced osteolysis since such a significant effect had been observed in task 1. MMP-2 deficient mice showed a difference in the amount of trabecular bone loss but not in the number of active osteoclasts, we decided to investigate the potential role of stromal MMP-2 in osteoclast function and migration. Unfortunately, the *ex vivo*

calvaria model has not recapitulated the observed effects i*n vivo* with respect to tumor induced osteolysis. Therefore, we are taking *in vitro* as well as *in vivo* approaches to test the role of stromal MMP-2 in osteoclast migration and function. We will isolate osteoclasts from wild-type and MMP-2 deficient mice and test their ability to resorb dentin slices. Recent studies demonstrated that the lack of host MMP-2 leads to a severe defect in osteocytic canicule network [14]. Mosig and colleagues also showed that osteoblasts deficient for MMP-2 present a decreased proliferation rate compared to the controls [15]. As osteoclast activation is critically dependent on proper osteoblast function, we decided to investigate further the impact of MMP-2 on osteoblast function/osteoclast activation. We will also assess the ability of wild-type and MMP-2 lacking osteoclasts to properly respond to osteoblast signaling. We will isolate osteoblasts from calvaria of wild-type or MMP-2 deficient mice and co-culture them with osteoclasts isolated from tibias of wild-type or MMP-2 null mice. We will test the ability of MMP-2 null osteobasts to properly activate osteoclasts. Since we observed an effect of stromal MMP-2 in tumor growth and survival, we will focus on factors in the bone that can control tumor growth such as TGF-β or insulin growth factor (IGF).

Key research accomplishement

1. Demonstration that stromal MMP-2 has a significant role in mammary tumor growth in the bone *in vivo*.
2. Stromal MMP-2 contributes to bone resorption by affecting osteoclast/osteoblast function
3. Host-derived MMP-9 does not affect the growth of mammary tumor in bone in the context of intra-tibial injection.
4. Host MMP-7 contributes to mammary tumor growth in the bone and osteolysis *in vivo*.

Reportables outcomes:

- **American Society of Matrix Biology biennial meeting**, October 2006, poster presentation
"Stromal MMP-2 promotes mammary tumor-induced osteolysis *in vivo*"
Sophie Thiolloy, Conor C. Lynch, Michelle D. Martin, Barbara Fingleton, Lynn M. Matrisian

- **6th Annual Host -tumor Interactions and Cancer Biology joint retreat**, November 2006, oral presentation, Oral talk award (1st place)
"Stromal MMP-2 contributes to mammary tumor induced osteolysis *in vivo* "
Sophie Thiolloy, Conor C. Lynch, Michelle D. Martin, Barbara Fingleton and Lynn M. Matrisian

- **Cancer Induced Bone Disease meeting,** San Antonio, December 2006, poster presentation
"Stromal MMP-2 promotes mammary tumor-induced osteolysis *in vivo* "
Sophie Thiolloy, Conor C. Lynch, Michelle D. Martin, Barbara Fingleton, and Lynn M. Matrisian

- **7th Annual Host -tumor Interactions and Cancer Biology joint retreat**, November 2007, 2 poster presentations
"The *in vivo* impact of host MMP-2 to mammary tumor induced osteolysis"
Sophie Thiolloy, Conor C. Lynch, Barbara Fingleton, Lynn M. Matrisian
"The differential contributions of host MMP-7 and MMP-9 on mammary tumor induced osteolysis"
Sophie Thiolloy, Ginger E. Holt, Jennifer Halpern, Herbert S. Schwartz, Gregory R. Mundy, Lynn M. Matrisian and Conor C. Lynch

Conclusions and future directions

Over the first year of our studies, we showed the significant importance of stromal MMP-2 in the growth of mammary tumor cells in bone. We demonstrated that stromal MMP-2 contributes to the growth and the survival of the tumor cell in the bone and does not affect the activation of osteoclasts. Over the second year, we

developed molecular tools such as wild-type and MMP-2 null FVB GFP expressing mice and a cell line expressing both luciferase and DsRed2 to investigate further the role of host-derived MMP-2 in mammary tumor survival in the bone. We also determined that stromal MMP-7 contributes to mammary tumor growth in the bone and osteolysis *in vivo*. Although MMP-2 and MMP-9 present a high homology and several common substrates, we have determined that in our model of mammary tumor induced osteolysis, MMP-9 does not contribute to the growth of the tumor and the bone resorption. So far, we showed differential effects of three specific MMPs in mammary tumor growth induced osteolysis, emphasizing the importance to determine the role of specific MMPs in breast cancer induce bone resorption to hopefully lead to the development of better therapeutics.

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